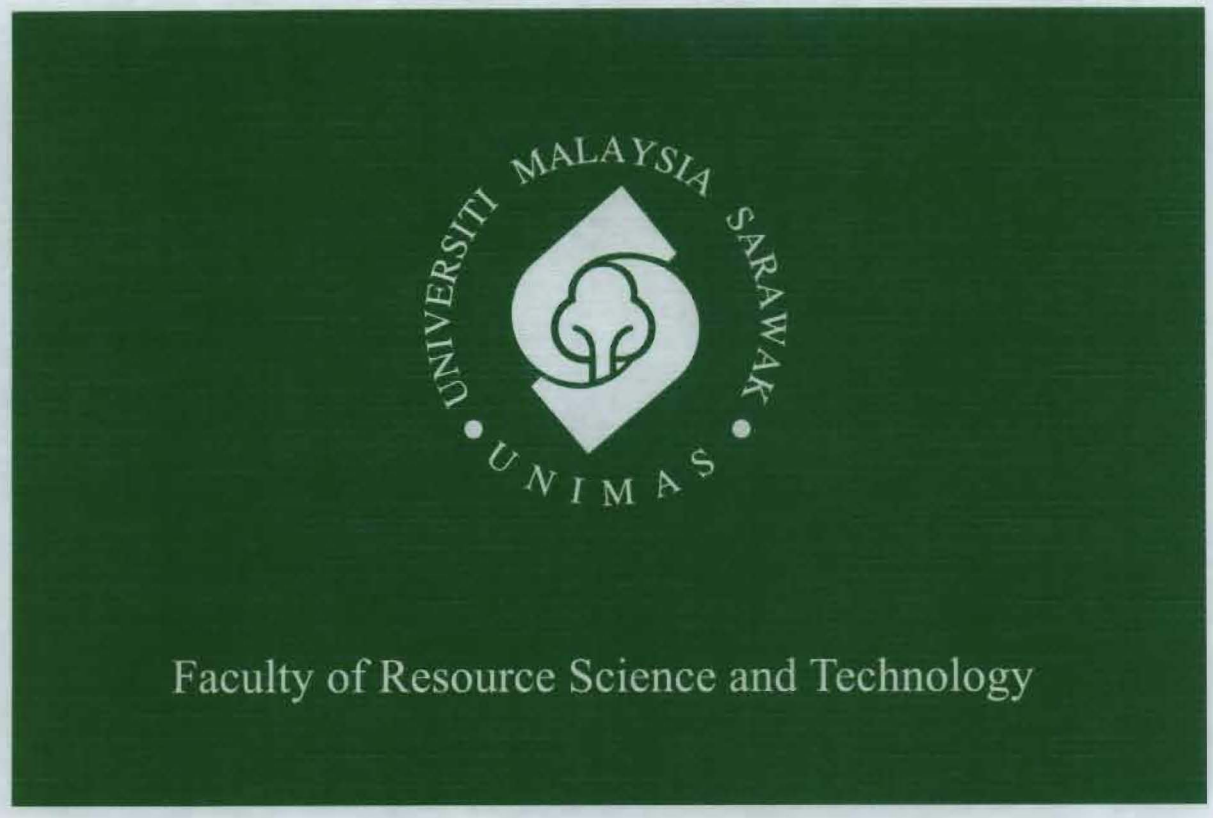

ISOLATION AND IDENTIFICATION OF CAUSAL DISEASE OF *EUCALYPTUS PELLITA*

Muhammad Safril Bin Abdul Rahim

Bachelor of Science with Honours
(Plant Resource Science and Management)
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ISOLATION AND IDENTIFICATION OF CAUSAL DISEASE OF EUCALYPTUS PELLITA

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This project report is submitted in partial fulfilment of the requirements for the Degree of Bachelor of Science with Honours
(Plant Resource Science and Management)

Department of Plant Science and Environmental Ecology

Faculty of Resource Science and Technology

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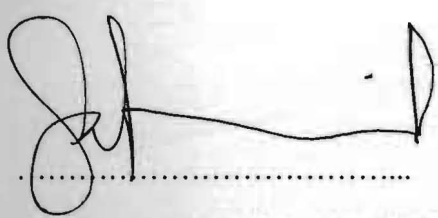
DECLARATION

I hereby declare that the Final Year Project Report is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at UNIMAS or other institutions of higher learning.



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In the name of Allah, the Most Beneficent, the Most Merciful.

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LIST OF ABBREVIATIONS

°C	Celsius
%	percentage
µg	microgram
ml	millilitre
g/L	gram per litre
w/v	weight per volume
ANOVA	Analysis of Variance
HCl	Hydrochloric acid
KOH	Sodium hydroxide
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
MEA	Malt Extract Agar
CMA	Corn Meal Agar
RAPD	Random Amplified Polymorphic DNA

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Isolation of Fungi and Identification of Causal Disease of *Eucalyptus pellita*

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ABSTRACT

Eucalyptus plantation in Malaysia is still new and need a lot of attention and care especially on the prevention against the disease infection. Disease infection in the timber tree species of the *Eucalyptus pellita* negatively impacts the timber yield throughout the world and affecting the production. A study was conducted in Sempadi Forest Plantation in Bau area, the tissues from the *Eucalyptus pellita* were collected, the disease description were made based on the symptoms, and the pathogen were isolated from the leaves and identified for their species based on the morphology and the molecular approach. The common disease symptoms were brown leaf spots, yellow leaf spots, reddish brown leaf spots, yellowish spots, whitish grey spots and also some discolouration of the leaf tips. The fungi isolated were *Cladosporium* sp., *Pestalotiopsis* sp., *Aspergillus niger*, *Aspergillus flavus*, *Trichoderma* sp., *Botryodiplodia theobromae* and Guignardia sp. Three physiological tests were conducted on the fungi isolated which were on different artificial media, temperature and pH. Three different media were used in the tests which were the MEA, CMA, and PDA. There were significant differences on the average growth of the fungi and the MEA was the best media for *Botryodiplodia theobromae* and *Trichoderma* sp. while PDA was the best for *Pestalotiopsis* sp. The tested temperature was at 15, 20, 25, 30, 35, 40°C. The fungi isolated grew the best at the moderate temperature which was in the range of 25-30°C. The pH test were conducted with the tested pH were at pH 3, 4, 5, 6, 7, 8 and the pH did not have a significant impact to the growth for most of the fungi. Pathogenicity test has been carried out on the detached leaf of the *E. pellita* by infecting it with *Cladosporium* sp. A serious study on the planted forest disease in Malaysia should be carried out on the wide range of timber species as a proper documentation on pathogen will resulting in the good prevention measures.

Keywords: timber, disease infection, pathogens, fungi, molecular approach

ABSTRAK

Perladangan eucalyptus di Malaysia adalah masih baru dan memerlukan banyak perhatian dan penjagaan terutamanya mengenai pencegahan terhadap jangkitan penyakit. Jangkitan penyakit dalam spesies pokok kayu Eucalyptus pellita negatif kesan hasil balak di seluruh dunia dan menjejaskan pengeluaran. Satu kajian telah dijalankan di Ladang Hutan Sempadi di kawasan Bau, tisu dari Eucalyptus pellita telah dikumpulkan, keterangan penyakit telah dibuat berdasarkan gejala, dan patogen yang telah diasingkan daripada daun dan dikenal pasti untuk spesies mereka berdasarkan morfologi dan pendekatan molekul. Gejala-gejala penyakit bintik daun coklat, bintik daun kuning, bintik daun coklat kemerahan, tompok kekuningan, bintik kelabu keputihan dan juga beberapa perubahan warna hujung daun. Kulat yang terpencil adalah Cladosporium sp., Pestalotiopsis sp., Aspergillus niger, Aspergillus flavus, Trichoderma sp., Theobromae Botryodiplodia dan Guignardia, sp. Tiga ujian fisiologi telah dijalankan ke atas kulat terpencil yang berada di media yang berlainan suhu tiruan, dan pH. Tiga media yang berbeza digunakan dalam ujian yang MEA, CMA, dan PDA. Terdapat perbezaan yang signifikan kepada pertumbuhan purata kulat dan MEA adalah media terbaik untuk Botryodiplodia theobromae dan Trichoderma sp. manakala PDA adalah terbaik untuk Pestalotiopsis sp. Suhu yang diuji adalah pada 15, 20, 25, 30, 35, 40 ° C. Kulat yang terpencil berkembang terbaik pada suhu sederhana yang berada dalam julat 25-30 ° C. Ujian pH telah dijalankan dengan pH yang diuji adalah pada pH 3, 4, 5, 6, 7, 8 dan pH tidak mempunyai impak yang besar kepada pertumbuhan kulat. Ujian patogenisiti telah dijalankan ke atas daun berkembar pellita E. dengan menjangkiti ia dengan Cladosporium sp. Satu kajian serius terhadap penyakit hutan yang ditanam di Malaysia perlu dijalankan dalam pelbagai spesies kayu sebagai dokumentasi yang sepatutnya pada patogen akan menyebabkan langkah-langkah pencegahan yang baik.

***Kata kunci**: kayu, jangkitan penyakit, patogen, kulat, pendekatan molekul*

CHAPTER ONE

INTRODUCTION

1.1 Research background

According to Old et al (2003), the *Eucalyptus* spp. is of second global importance in the plantation tree programmes after the pines species. Data published by the FAO (1995) and GIT Forestry Consulting (2008) stated that, in 1995 there were 683 000 ha of normal Eucalypts plantation in the Southeast Asian region and that was not including the equivalent of about 2.0 million ha as boundaries trees around the field and also the scattered trees. In the year 2008, there is a huge increment of the plantation area of the *Eucalyptus* spp. in those countries with the total of approximately 1.5 million ha (Table 1).

Table 1. Estimated areas of Eucalyptus plantations in the South-East Asian region in 1995 until the year of 2008. Sources: FAO and GIT Forestry Consulting.

	Plantation area of the Eucalyptus by year (ha)	
Country	**1995**	**2008**
Indonesia	80 000	128 000
Malaysia	8 000	19 000
Myanmar	40 000	76 189
Philippines	10 000	189 000
Thailand	195 000	500 000
Vietnam	350 000	586 000
Total	683 000	1 498 189

From the above table it can be concluded that there is a lot of demand for Eucalypts resulting in an increase of the plantation area of this species from 1995 to 2008. The statistics give a clear view that the *Eucalyptus* spp. are in demands for their products yearly.

In Malaysia, there is an effort to plant the *Eucalyptus* spp. in the large scale plantation. From their establishment in Malaysia in the year of 1893 (FAO, 1979) as the ornamental trees, *Eucalyptus* spp. has been introduced to the forestry plantation in the small scale beginning in the year of 1931with the plantation area of 40 ha in Cameron Highlands Forest Reserves for the proposed used as timber and fuel wood and in the year of 1995 the area increase by the number of 8 000 ha followed by the year of 2008 by 19 000 ha (GIT Forestry Consulting, 2008; FAO, 1995; Sulaiman, 1993) (Figure 1). In Sabah, the forestry plantation of the *Eucalyptus* spp. was initiated by the private company in the year of 1974 (Tan, 1987).

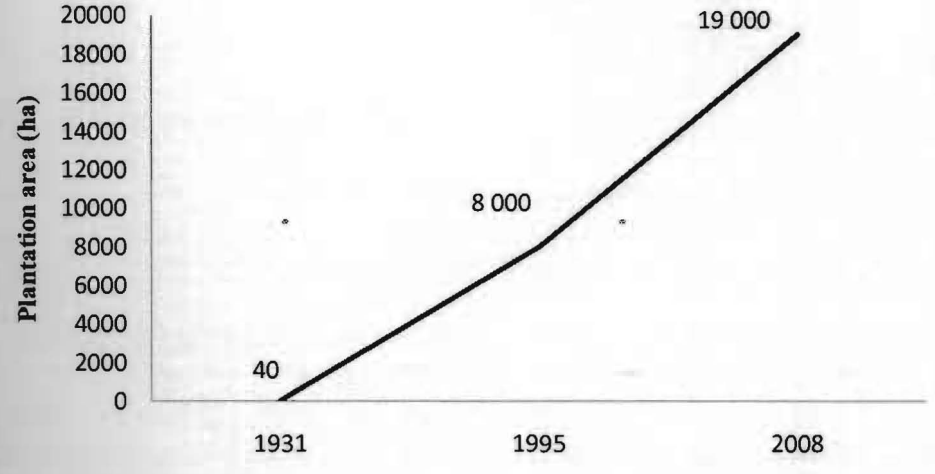

Figure 1: Plantation area of *Eucalyptus spp.* in Malaysia from the year 1941-2008

The statistical value shown by the above graph showed that there is an increase of the planted *Eucalyptus* spp. area in Malaysia. It can be concluded that the *Eucalyptus* spp. have a high demand in Malaysia and it can be made as one of the timber sources.

Based on studies made by researchers in the other countries, the *Eucalyptus* spp. is susceptible and vulnerable to the disease infection. Thus it can disrupt the growth performance of the tree and reduce the quality and the productivity of the tree. As an example, in many African countries, disease has negatively impacted the plantations of the Eucalypts (Gezahgne, 2010).

This research is aims to identify all the possible pathogen that associated with the Eucalyptus in Malaysia especially in Sarawak. *Eucalyptus* spp. plantation is still new in Malaysia and need an extensive attention in the prevention of the disease.

1.2 Problem statement

The number of the *Eucalyptus* spp. plantations in the world especially in the Asian region and Malaysia is increasing yearly. A high rate of plantation productivity is needed in order to meet the demand of the worldwide population for the products of the Eucalypts trees. The large-scale growth of the *Eucalyptus* spp. plantations will cause the trees to be more vulnerable to disease and thus will reduce the quantity and quality of the products. In Malaysia, *Eucalyptus* spp. is still in the early stage of the plantation and there is still a lack of information on the disease of the Eucalypts in the Malaysian environment and climate. Many new diseases will develop in time and the inability to identify it will cause more problems in handling the infection. So there is a need to rapidly isolate and identify the causal

disease that mainly comes from an infection so that the first step for the prevention can be taken. This step must be done in order to reduce the impact of the infection, reducing the cost for the control and also to save this valuable species from destruction. The morphological and molecular approach to fungal identification and also the physiological characteristics of the pathogen will be assessed in order to characterize the potential pathogen associated with diseases of the Eucalypts species in Malaysia.

1.3 Objectives

The objectives of this research are:

a) To identify the causal disease of the Eucalyptus species based on the morphological characteristics of the fungi and also by using the molecular technique.
b) To study the physiological characteristics of the fungi towards different temperatures and pH level.
c) To determine the pathogenicity of the fungi on the Eucalyptus species.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of eucalyptus

Eucalyptus is an enormous genus comprising of more than 800 species and two third of them can be found in Australia (Barclay, 2004). The genus of Eucalypts belongs to discrete groups which can be classify into five major subgenera which are Blakella, Corymbia, Eudesmia, Gaubaea, Idiogenes, Monocalyptus and also Symphyomyrtus as introduced by Pryor and Johnson (1971). The eucalyptus comes from the family Myrtaceae which is commonly found in Australia, South America and Asia and it is closely related to the syzygium genus (Lyne, 1996).

In Malaysia, there are two species of Eucalyptus that are commonly being planted which are *E. Deglupta* and *E. Grandis* (Sabah Forestry Department, 2006). The Eucalyptus is abundantly planted in the plantations in Sabah, Pahang and Negeri Sembilan and some of them are planted as the ornamental plant (Sulaiman, 1993).

2.2 Botanical description

Lyne (1996) summarized the *Eucalyptus* as it exhibiting a variety of habits such as it can be a shrub, mallees or trees and it mostly occur as forest and woodland trees. The bark can be classified into two categories which are the smooth and rough bark and in some species it is very hard. The flower is an umbellaster which

usually simple and axillary, compound and axillary and compound and terminal whereas the fruit is a woody hypanthium that enclosed the base and sides of the capsules and it may vary in shape according to their species.

2.3 Origin and distribution of eucalyptus

Species of the genus eucalyptus are commonly known as eucalypts throughout the world, although in Australia they often called gum trees because of the gum (kino) that exudes from the trunk of older trees (Zacharin, 1978). Eucalyptus originated from Australia (Coppen, 2002). According to Turnbull (1999), the distribution of natural eucalypts forest is widely spreading among the Australian country, whereas there are a lot of countries in the world such as the Americas and South Africa that plant the *Eucalyptus* in the large-scale that include the various of species. In Sarawak, there are only 0.4 ha have been planted with *Eucalyptus* spp. (Sulaiman, 1993).

2.4 Importance of eucalyptus

Young trees are a source of paper pulp, charcoal and fuel wood, poles, mining timber, and fibreboard; mature trees within species provide strong and durable timber, and all sizes are capable of use for other forest products such as volatile oils for pharmaceutical and industrial uses, and honey (Penfold and Willis, 1961; Jacobs, 1981; Hillis and Brown 1984; Boland et al, 1991). Eucalypts provides sawn timber, plywood, fibreboard, mine props, pulp for paper and rayon, poles,

firewood and charcoal, essential oils, honey, shade and shelter (Hillis and Brown, 1978). Less conventional uses include the production of plant growth regulator, tannin extracts, industrial chemical additives, adhesives and fodder additives (Song, 1992).

2.5 Forest plantation diseases

According to Callan (2001), tree diseases cover the wide range of pathogenic infection, abnormalities, disturbance of the normal structure and growth of the tree and he defined tree disease as the deleterious effects resulting from injurious agents other than fire and insect damage and it usually develop from the complex interaction between the susceptible tree, predisposing environment condition or infectious agents such as fungi. There are many diseases that infect the stem, root and leaves. Cryphonectria canker caused by *Cryphonectria cubensis*, canker and dieback is caused by *Botryosphaeria* spp., vascular wilt of *Eucalyptus* caused by *Ceratocystis fimbriata*, pink disease caused by *Erythricium salmonicolor* and Leaf blotch caused by *Mycosphaerella* spp. are examples of diseases in commercial *Eucalyptus* plantations (Gezahgne, 2010). In Malaysia, leaf fungus disease wiped out plantations of *Eucalyptus camaldulensis* in Malaysia and the incidence of heart rot has resulted in a drastic, temporary slowdown of plantation establishment in Peninsular Malaysia (Lee, 1993).

According to Davis (2002), the serious fungal infestation will occur when the density of leaf of the Eucalyptus is high and the damage due to the infestation can spread very rapidly, destroying large areas of leaves within a few days if not

checked. Harrison et al (2003) stated that, the *Ganoderma* species has been reported affecting the *Eucalyptus* spp. According to Philiips (1994), the most common diseases for the Eucalypts are caused by the fungi of:

1. *Mycosphaerella* spp. (Crinkle Leaf Disease)
2. *Aulographina eucalypti* (Corky Leaf Spot)
3. *Pseudocercospora eucalyptorum*
4. *Septoria pulcherrima*
5. *Seimatosporium* spp. (Angular Leaf Spot)

2.6 Pathogenic fungi

Pathogenic fungi can be described as the virulent fungi which produce the inoculums which is the spores to infect the host. The classification and identification of fungi, unlike other important pathogens such as bacteria or viruses, relies mainly on morphological criteria. The identification of the fungi is a complex process that usually requires microscopic examination and extensive knowledge of the taxonomy of the fungus and most of the tree specialists and arborist can recognize most fungus diseases by the appearance of the tree section (the symptoms) (Tattar, 1989).

According to Callan (2001), fungi associated with the tree disease can be classified into several classes which are:

1. Water molds: Oomycetes
2. Sac fungi and molds: Ascomycetes
3. Mushrooms and conks: Hymenomycetes
4. Rusts: Urediniomycetes (Basidiomycota)

2.7 Forest plantation pathology principles

Callan (2001) stated that, there are several principles that is important in the forest pathology research which are:

1. Culturing - the suspected pathogen should be obtained in a pure, artificial culture, where possible
2. Pathogenicity study - according to Kochs' Postulates (see below), the organism should be proven to be the cause of an infectious disease.
3. Life cycle - all spore states should be identified and studied and related to host range and phenology
4. Conditions for infection - the general physiology and requirements for spore germination, penetration, and so on should be elucidated.

Koch Postulates:

1. Show constant association of the organism with the disease.
2. Isolate the organism in culture from the diseased plant.
3. Inoculate a healthy plant from the culture and produce the same disease.
4. Re-isolate the same organism from inoculated plants.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research material

Diseased tissues such as leaves, branches and roots from the Eucalyptus tree were used in this research. Fresh infected samples of the *Eucalyptus pellita* species were collected from the Sampadi Forest Plantation in Kuching, Sarawak.

3.2 Disease description

The descriptions of the disease on the infected tissues were made. All the symptoms such as the presents of the lesion on the leaves were identified. The early identification of the disease was made based on the available references.

3.3 Pathogens isolation on the Potato Dextrose Agar

To identify the potential pathogens that associated with the diseases, the pathogens were isolated on the media. Potato dextrose agar (PDA) was as a media for the isolation of the pathogens.

The samples of the Eucalyptus leaves were cut into 100 small square segments which have the size of 2mm x2mm. The tissues were cut at the margin of the lesion or between the healthy and the infected site by using the sterile scalpel to

prevent any other contamination. Then, the tissue segments were agitated in the 10% concentration of sodium hypochlorite, and the tissues segments were rinsed into the sterilized distilled water, three times for five minutes in each session. The segments were blotted dry using the sterilized filter paper.

The segments were put onto the media, with ten tissue segments for each Petri dish. The segments plated were incubated in the room temperature. The observation was made daily until no new species of fungi can be found grow from the plated plant tissues in the Petri dishes. The different types of the fungi were observed and the potential pathogen was identified during the incubation period. Percentage occurrence of the fungi associated with the tissue segments was recorded.

A pure culture was prepared for the further studies. The pure cultures were prepared by inoculating the morphologically different hyphal tips from the isolated fungi into a new PDA media. The inoculated media were incubated at the room temperature.

A stock culture was prepared. A small block of agar containing the mycelia from the four to seven days culture were cut from the media and it were kept in a small bottle containing the sterilized water and it were incubated at the temperature of 4°C.

The identification of the fungi was made based on the morphological characteristics and also the molecular approach.

3.4 Identification of fungi using morphological characteristics

To determine their morphological characteristics, the fungi were observed under the compound microscope. In order to make it more visible under the microscope, Acid fuchsin which is red in colour was used. The morphological characteristics that was observed and identified are the vegetative hyphae and the shape of the conidia. Picture of all of these characteristics was capture using digital camera for further record and identification. Reference literature used to aid the identification.

3.5 Identification of fungi using molecular approach

All selected fungal isolated were grown on the Malt Extract Agar (MEA) for four to seven days. For a better DNA yield, the mycelial mat from the two weeks old cultures in the Potato Dextrose Broth (PDB) also used for the DNA extraction. 0.3 g of the mycelium of the fungi was scraped off from the surface of the MEA. It was grinded in liquid nitrogen using a mortar and pestle until the dry powdery extract was formed. The mycelium powder was transferred into a sterile 1.5 ml centrifuge tube. The 500 µl DNA extraction buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA; 3 M NaOAc; and 1% SDS) was preheated in the 60° C water bath and it was cooled down to the room temperature. Then, the extraction buffer was added into the centrifuge tube and the mixture was incubated for 25 minutes at 55° C. After the incubation, the mixture was vortexed to prevent the coagulation and clumping of the sample. 500 µl of phenol/ chloroform/ isoamylalcohol (25:24:1) was added into the mixture and the centrifuge tube was centrifuged at 13 000 rpm for five minutes. The top layer of supernatant resulting from the high speed centrifugation was transferred into a new centrifuge tube and 500 µl of